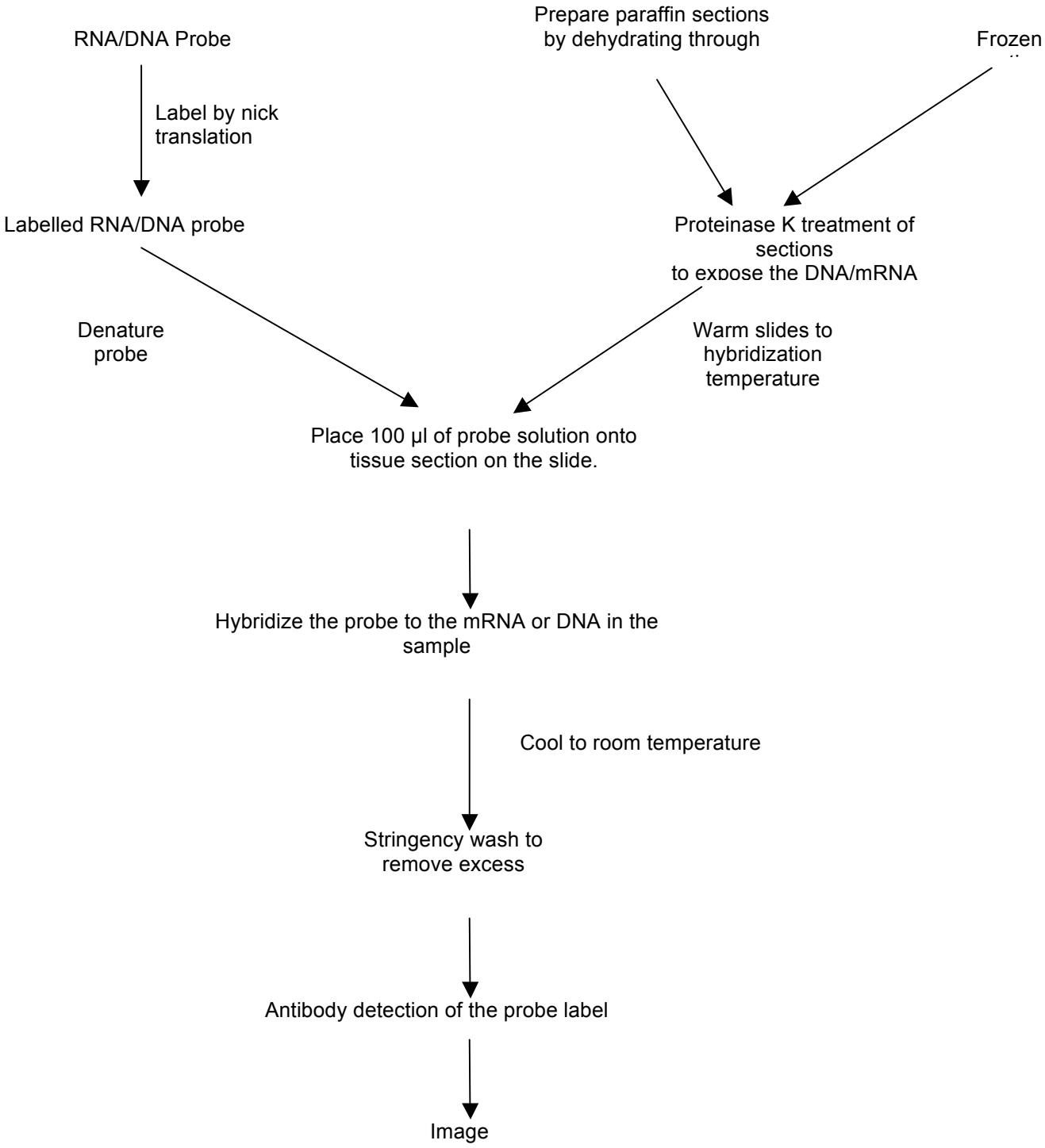
In situ hybridization

In situ hybridization indicates the localization of gene expression in their cellular environment. A labeled RNA or DNA probe can be used to hybridize to a known target mRNA or DNA sequence within a sample. This labeled RNA or DNA probe can then be detected by using an antibody to detect the label on the probe. The probes can therefore be used to detect expression of a gene of interest and the location of the mRNA.



TIPS:

Storage of samples; RNA preservation in the sample
Preserving DNA is easy because it is a highly stable molecule. However, preserving RNA is much more difficult due to presence of RNase enzyme. This may be found on glassware, reagents and on the operator and their clothing. RNase will quickly destroy any RNA in the cell or the RNA probe itself. Therefore users must ensure they use sterile techniques, gloves, and solutions to prevent RNase from contaminating and destroying the probe or tissue RNA.

General sample storage when using frozen sections
For good results on older slides, the slides should not be stored dry at room temperature. Store either in 100% ethanol at -20°C, or place in a plastic box covered in saran wrap at -20° or -80°C. Slides stored in this way can be used for several years.

Choice of probe

RNA probes
RNA probes should be between 250 to 1500 bases in length. Probes approximately 800 bases long exhibit the highest sensitivity and specificity. Ideally transcription templates should allow for transcription of both probe (antisense strand) and negative control (sense strand) RNAs. Cloning into a vector with opposable promoters will achieve this. Circular templates must be linearized before making a probe. PCR templates can also be used for this purpose.

DNA probes:
DNA probes can also provide high sensitivity to RNA probes. However, they do not hybridize as strongly to the target mRNA molecules. Therefore, formaldehyde should not be used in the post hybridization washes when using DNA probes.

Specificity of the probe is extremely important. If the exact nucleotide sequence of the mRNA or DNA in the cell is known, a precise complementary probe can be designed. If over 5 percent of the base pairs are not complementary, the probe will hybridize only loosely to the target sequence. This means the probe is more likely to be washed away during wash steps and detection steps and the probe may not be detected, or only some of the sites may be detected and the labeling will not be an accurate representation.

DIG (Digoxigenin) labeled RNA probe In situ hybridization protocol

The protocol shown here describes the use of DIG labeled single stranded RNA probes to detect expression of the gene of interest in paraffin embedded section. It is a highly sensitive technique.

1. Deparaffinization

If using formaldehyde fixed paraffin embedded sections.
For frozen sections, please start at section 2

Before proceeding with the staining protocol, the slides must be deparaffinized and rehydrated. Incomplete removal of paraffin can cause poor staining of the section.

Materials and reagents
- Xylene
- 100% ethanol
- 95% ethanol

Method
Place the slides in a rack, and perform the following washes:

1. Xylene: 2 x 3 mins.

2. Xylene 1:1 with 100% ethanol: 3 mins.

3. 100% ethanol: 2 x 3 mins.

4. 95% ethanol: 3 mins.

5. 70 % ethanol: 3 mins.

6. 50 % ethanol: 3 mins.

7. Running cold tap water to rinse.

Keep the slides in the tap water until ready to perform antigen retrieval. At no time from this point onwards should the slides be allowed to dry. Drying out will cause non-specific antibody binding and therefore high background staining.

2. Antigen retrieval

Digest with 20 µg/ml proteinase K in prewarmed 50 mM Tris for 10 to 20 min 37°C. The time of incubation and concentration of proteinase K may require some optimization.

The concentration of proteinase K and the incubation time for this step will require optimization. We can recommend trying a proteinase K titration experiment to determine the optimal conditions. Insufficient digestion will result in a reduced hybridization signal. Over digestion will result in poor tissue morphology, making localization of the hybridization signal very difficult. The concentration of Proteinase K needed will vary depending upon the tissue type, length of fixation, and size of tissue.

3. Rinse slides 5 times in distilled water.

4. Immerse slides in ice cold 20% (v/v) acetic acid for 20 seconds. This will permeabilize the cells to allow access to the probe and the antibody.

5. Dehydrate the sections by washing for approximately 1 min each wash in 70% EtOH, 95% EtOH and 100% EtOH then air dry.

6. Add 100 µl hybridization solution to each section.

Reagent	Final conc	Amount to use per 1 ml of solution
Formamide	50%	500 µl
Salts	5x	250 µl
Denhardt's solution	5x	100 µl
Dextran sulphate	10%	200 µl
Heparin	20 U / ml	10 µl
SDS	0.1%	10 µl

Salt solution:
4 M NaCl
100 mM EDTA
200 mM Tris-HCl pH 7.5
100 mM $NaH_2PO_4.2H_2O$
100 mM NaH_2PO_4

Denhardt's solution (100x):
10 g Ficoll
10 g PVD (polyvinylpyrrolidine)
10 g BSA (Bovine Serum Albumin)
500ml sterile dH_2O

7. Incubate the slides 1 hour in hybridization chamber at the desired hybridization temperature. Typical hybridization temperatures range between 55 and 62°C (for more details, see notes with section 9 below)

8. Dilute the probes in hybridization solution ready in PCR tubes. Heat for 95°C for 2 min on a PCR block. This will dehybridize the RNA or DNA probe. Chill on ice immediately to prevent rehybridization.

9. Drain off the hybridization solution. Add 50 to 100 ul per section of diluted probe (ensure the entire sample is covered). Incubate in the hybridization chamber 65°C overnight. Whilst incubating, the sample on the slide can be covered with a coverslip to prevent evaporation.

During this step, the RNA probe will hybridize to the corresponding mRNA, or the DNA probe will hybridize to the corresponding cellular DNA.

The hybridization temperature will require optimization depending on the sequence of the probe used, as well as the cell / tissue type. This temperature should be optimized for each tissue type analyzed. Hybridization temperatures used range from 55 to 62°C.
The optimal hybridization temperature for the probe depends on the percentage of bases present in the target sequence. The concentration of cytosine and guanine in the sequence are an important factor.

10. Stringency washes:

Solution parameters such as temperature, salt and/or detergent concentration can be manipulated to remove any non-identical interactions (i.e. only exact sequence matches will remain bound).

To prepare 1 liter of 20 x SSC:
For 1 liter:
175.3 g NaCl (3 M)
88.2 g Na citrate
800 ml sterile dH_2O
Adjust to pH 5 using Citric acid, top up to 1 liter and then autoclave.

Wash 1 50% formamide / 2 x SSC
3 x for 5 min, 37- 45° C.

To wash away any excess probe and the hybridization buffer. Higher temperatures (up to 65°C) can be used for short periods of time, but this can wash off too much of the hybridiized probe RNA / DNA if left for too long.

Wash 2 0.1-2 x SSC
3 x for 5 min, 25°C to 75°C.

This step removes non-specific and/or repetitive DNA / RNA hybridization. The less concentrated the salt solution and the longer the duration of the wash and the temperature, the higher the stringency and the more DNA / RNA will be removed.

Optimization of temperatures for stringency washes can be difficult to work out, but the following guidelines can help:

Very short DNA/RNA probes (0.5-3 kb) or very complex probes, the washing temperature should be lower (up to 45°C) and the stringency lower (1x-2 x SSC).

Single-locus or large probes, the temperature should be around 65°C and the stringency high (below 0.5 x SSC).

The temperature and stringency should be highest for repetitive probes (such as alpha-satellite repeats).

11. Wash twice in MABT (maleic acid buffer containing Tween 20) for 30 min at room temperature.

MABT is gentler than PBS and is more suitable for nucleic acid detection.

5 x MABT stock:
500 ml maleic acid pH 7.5
750 mM NaCl
0.5% v/v Tween 20
pH 7.5

12. Dry the slides.

13. Transfer to a humidified chamber and add 200 µl blocking buffer to each section (MABT + 2% BSA, milk or serum). Block for 1 to 2 hours, room temperature.

14. Drain off the blocking buffer. Add the anti-'label' antibody the required dilution in blocking buffer. Check the antibody datasheet for a recommended concentration. Incubate for 1 to 2 hours at room temperature.

15. Wash slides 5 times with MABT, 10 minutes for each wash, room temperature.

16. Wash the slides 2 x for 10 min room temperature with prestaining buffer (100 mM Tris pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$).

17. Fluorescence – please go next to step 18.

18. Other – return slides to humidity chamber and follow manufacturer's instructions for color development.

19. Rinse slides in distilled water.

20. Air dry the slides for around 30 minutes. Wash in 100% ethanol, then air dry thoroughly.

21. Mount using DePeX mounting solution.